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Reaction products of hexamethylene diisocyanate vapors with "self" molecules in the airways of rabbits exposed via tracheostomy

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Abstract

1. Hexamethylenediisocyanate (HDI) is a widely used aliphatic diisocyanate and a well-recognized cause of occupational asthma.
2. "Self" molecules (peptides/proteins) in the lower airways, susceptible to chemical reactivity with HDI, have been hypothesized to play a role in asthma pathogenesis and/or chemical metabolism, but remain poorly characterized.
3. This study employed unique approaches to identify and characterize "self" targets of HDI reactivity in the lower airways. Anesthetized rabbits free breathed through a tracheostomy tube connected to chambers containing either, O_2, or O_2 plus ~200 ppb HDI vapors. Following 60 minutes of exposure, the airways were lavaged and the fluid was analyzed by LC-MS and LC-MS/MS.
4. The low-molecular weight (<3 kDa) fraction of HDI exposed, but not control rabbit bronchoalveolar lavage (BAL) fluid identified 783.26 and 476.18 *m/z* $[M+H]^+$ ions with high energy collision-induced dissociation (HCD) fragmentation patterns consistent with bis glutathione (GSH)-HDI and mono(GSH)-HDI. Proteomic analyses of the high molecular weight (>3 kDa) fraction of exposed rabbit BAL fluid identified HDI modification of specific lysines in uteroglobin (aka clara cell protein) and albumin.
5. In summary, this study utilized a unique approach to chemical vapor exposure in rabbits, to identify HDI reaction products with "self" molecules in the lower airways.

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Declaration of interest

No potential conflict of interest was reported by the authors.

Keywords

Exposure; glutathione; hexamethylene diisocyanate; tracheostomy

Introduction

Diisocyanates are reactive chemicals with important uses in many different industries (Allport et al., 2003). Aliphatic diisocyanates are used to make elastomers and polyurethane coatings that help protect against corrosion, abrasion and damage from ultraviolet light (Ulrich, 1996). Global production of aliphatic diisocyanates exceeds 100 000 metric tonnes/year (DOW, 2010), with usage in civilian, for example, autobody clear coat (Fent et al., 2009; Pronk et al., 2007; Reeb-Whitaker et al., 2012; Sparer et al., 2004) and military sectors, for example, chemical agent-resistant coating (CARC) for aircraft, tanks, etc. (CARC 2000; Carlton & England, 2000; Kycia et al., 2012; LaPuma & Bolch, 1999; Wisnewski et al., 2012). Contemporary formulations generally consist of HDI oligomers with limited respirability (unless sprayed or heated) along with more volatile monomeric HDI and sometimes isophorone diisocyanate (IPDI) (Fent et al., 2009; Reeb-Whitaker et al., 2012; Thomasen et al., 2011). Clinical studies suggest monomeric HDI, its oligomers and IPDI all can cause asthmatic responses (Clarke & Aldons, 1981; Vandenplas et al., 1993) and many advisory agencies such as the ACGIH®, the United States of America's National Institute of Occupational Safety and Health, and the German Commission for the Investigation of Health Hazards of Chemical Compounds in the Work Area have recommended occupational exposure limits of 5 ppb as an 8-h time-weighted average and/or 20 ppb for short time periods (German-MAK-HDI, 2002; GERMAN-MAK-IPDI, 2002; Rom & Markowitz, 2007). However, the Occupational Safety and Health Administration for the United States and equivalent agencies in many other countries, have not established, nor enforce, occupational exposure limits for aliphatic diisocyanates (OSHA, 2012; Rom & Markowitz, 2007).

Despite recognition of HDI's ability to cause allergy/asthma, the mechanisms of disease pathogenesis remains unclear, hampering efforts at disease recognition, prevention and treatment (Redlich & Karol, 2002). Much uncertainty persists regarding the fate of HDI inhaled into the airways, and how it initiates airway pathology. One leading hypothesis is that HDI and other diisocyanates' react with "self" molecules (e.g. peptides/proteins), altering their conformation and creating neo-epitopes or other signals that stimulate the immune system (Karol, 2001; Kennedy et al., 1989, 1994; Lange et al., 1999; Pauluhn et al., 2006; Timchalk et al., 1994; Wisnewski et al., 2000). Albumin's role as a reaction target for diisocyanate is suggested by serology demonstrating antibodies that bind diisocyanate-conjugated albumin (but not other proteins) in exposed workers and the extraction of diamines (hydrolysis breakdown products of diisocyanates) from albumin in the peripheral circulation (Sabbioni et al., 2016; Sepai et al., 1995; Wisnewski et al., 2004). However, studies addressing the reactivity of diisocyanates with "self" molecules in the lower airways are ethically and logistically challenging to pursue in humans and have been limited to date (Redlich et al., 1997; Wisnewski et al., 2000).

Animal studies of HDI exposure to identify “self” reaction targets in the lower airway have been compromised by inherent species differences with humans; for example, rodents are obligate nose breathers and their upper airways are proportionally larger and possess a scrubbing effect or capacity for absorbing/capturing reactive vapors (Ferguson et al., 1988; Harkema et al., 2006; Kennedy et al., 1993; Morris & Buckpitt, 2009; Schroeter et al., 2013). Prior rat and guinea pig studies with several different radiolabeled monoand di-isocyanates demonstrated limited penetration into the lower airways and higher levels of upper airway and oral absorption (Ferguson et al., 1988; Gledhill et al., 2005; Kennedy et al., 1994; Schroeter et al., 2013).

The present study explored a unique approach for delivering HDI vapor to the lower airways of an animal (bypassing the upper airways and esophagus) and subsequent identification of “self” reaction targets. The methodology was adapted from a previously published approach for maintaining spontaneous breathing in tracheotomized rabbits (Xia et al., 2011), and an older study (Marek et al., 1995) demonstrating the utility of oral intubation for delivering toluene diisocyanate (TDI) vapor to rabbit lower airways. In the present study, an endotracheal tube was inserted surgically, which provided a ready port for obtaining lower airway fluid (via lavage) immediately after exposure and obviated the notorious challenges of oral intubation in rabbits (Gografe et al., 2003). Although rabbits have been largely neglected as a model for diisocyanate asthma, they offer several advantages over smaller animal models for the present investigation, including larger amounts of airway fluid for LC-MS/MS analyses and proteomics and greater homology of their albumin protein (a potential reaction target for HDI as described above) with humans (Wisnewski et al., 2000, 2004, 2010). The findings of the present in vivo study are discussed in the context of prior in vitro and in vivo investigations and the potential mechanisms underlying diisocyanate asthma pathogenesis.

Materials and methods

Animals

Four male New Zealand White rabbits supplied by Charles River (Wilmington, MA) weighing 2.7–4.3 kg and free of common rabbit pathogens were acclimated in the Yale animal care facility for 1–2 weeks prior to the study. The day of the study, rabbits were fasted for 2–3 h, and then, anesthesia was induced by intramuscular (IM) injection of ketamine 30 mg/kg and xylazine 3 mg/kg. Subsequently, ears were sterilely prepared and venous catheters were inserted into each ear. The left ear venous port was used for continuous rate infusion of ketamine 25 mg/kg/h and xylazine 2.5 mg/kg/h (in 0.9% saline) via an intravenous fluid infusion pump (approximately 4 mL/kg/h). The right ear venous port was used to deliver euthanasia solution. Buprenorphine 0.05 mg/kg was administered IM, and 0.5% marcaine (up to 2 mg/kg bupivacaine) was injected as a local anesthetic, prior to placement of the endotracheal tube via tracheotomy. A 3.5 or 4mm (inner diameter) cuffed with murphy eye endotracheal tube (Henry Schein® Animal Health; Dublin, OH) was utilized and secured with skin sutures. Two control rabbits (C1 and C2) were allowed to spontaneously breathe through a tracheostomy tube connected to an exposure chamber filled with O_2 (4 L/min). Two exposed rabbits (E1 and E2) spontaneously breathed through a

tracheostomy tube connected to an exposure chamber filled with O_2 containing 147–252 ppb HDI vapor (4 L/min) generated by passive diffusion from a 14-cm diameter pyrex glass petri dish containing 15mL of HDI, as previously described (Wisnewski et al., 2004, 2013b). Puriss grade HDI (CAS Number: 822-06-0) solution from Sigma–Aldrich (St. Louis, MO) was ≥99% pure by gas chromatography, with a refractive index of n20/D 1.453, and a density of 1.047 g/mL at 20 °C. HDI vapor concentration was monitored with an Autostep toxic gas monitor (GMD Systems; Pittsburgh, PA) and validated using ISO-CHEK methodology (Galson Laboratories; East Syracuse, NY). The HDI and control exposures were performed inside a Hamilton® SafeAire® fume hood (Fisher Hamilton LLC; Two Rivers, WI). Following one hour of control (O_2) or experimental (O_2+HDI vapor) exposure, the airways were lavaged with 60mL of 0.9% saline and animals were euthanatized by intravenous administration of Euthasol® (Virbac AH Inc; Fortworth, TX). All studies were performed in accordance with approval from our Institutional Animal Care and Use Committee and our Environmental Health and Safety Office. The animal care program is fully accredited by the Association for Assessment and Accreditation of Laboratory Animal Care.

Processing of rabbit airway fluid

Airway fluid was centrifuged at 400 *g* for 10 min to pellet the cells and debris. The supernatant was collected and separated into <3 kDa and >3 kDa fractions using Amicon Ultra 0.5-mL Centrifugal Filters Ultracel 3K (UFC500324) obtained from Merck Millipore Ltd (Billerica, MA).

LC-MS analysis of rabbit airway fluid using an Agilent Q-TOF system

Ten milliliter of the low molecular weight (<3 kDa) fraction of rabbit airway fluids was acidified with 0.06% trifluoroacetic acid (TFA) from Sigma/Aldrich and concentrated via solid-phase extraction using Sep-Pak Vac C18 Cartridges (WAT054955) from Waters (Milford, MA). Sep-Pak Vac C18 cartridges were eluted with stepwise increasing concentrations of acetonitrile. Three 0.5mL fractions were collected following elution with 20%, 60% and 100% acetonitrile. Each fraction was speedvacced to dryness, resuspended in HPLC/MS-grade water (Water Optima LC/MS-W6-4) from Fisher Scientific (Fairlawn, NJ) containing 0.1% formic acid from Sigma and subsequently analyzed on an Agilent G6550A Q-TOF system coupled to an Agilent 1290 Infinity LC system, using a rapid resolution HT Zorbax Eclipse Plus C18 column (2.1 × 50 mm, 1.8 μm), also from Agilent Technologies (Santa Clara, CA). Samples were mixed 1:1 in buffer A (water containing 0.1% formic acid) before loading and were eluted with 20% buffer B (acetonitrile containing 0.1% formic acid) over 2.5 min, increasing to 95% buffer B by 5 min and reverting back to 2% buffer B by 6 min. Positive-ion electrospray was performed using the following parameters: gas temp − 280 °C, gas flow − 11 L/min, nebulizer-40 psig, sheath gas temp − 350 °C, sheath gas flow-11, Vcap-4000 V, nozzle voltage-2000 V, fragmentor voltage− 175 V, skimmer voltage 65 V, octopole RF peak voltage 750 V, as previously described (Wisnewski et al., 2016). The data, acquisition range from 50 to 1700 *m/z*, were acquired and analyzed using Mass Hunter Workstation software from Agilent.

LC-MS/MS analysis of the <3 kDa fraction of rabbit airway fluid using a Thermo Scientific Q Exactive Plus

Concentrated fractions (<3 kDa) of airway lavage fluid that eluted from the Sep-Pak Vac C18 Cartridges with 20–60% acetonitrile were further analyzed by LC-MS/MS on a Thermo Scientific Q Exactive Plus (Waltham, MA) equipped with a Waters nanoAcquity UPLC system utilizing a binary solvent system (Buffer A: 100% water, 0.1% formic acid; Buffer B: 100% acetonitrile, 0.1% formic acid). Trapping was performed at 5 μL/min, 97% Buffer A for 3 min using a Waters Symmetry® C18 180 μm × 20mm trap column. Peptides were separated using an ACQUITY UPLC PST (BEH) C18 nanoACQUITY Column 1.7 μm, 75 μm × 250mm (37°C) and eluted at 330 nL/min with the following gradient: 3% buffer B at initial conditions; 5% B at 1 min; 35% B at 50 min; 50% B at 60 min; 90% B at 65 min; 90% B at 70 min; return to initial conditions at 71 min. MS was acquired in profile mode over the 300–1700 *m/z* range using 1 microscan, 70 000 resolution, AGC target of 3E6 and a maximum injection time of 45 ms. Data-dependent MS/MS were acquired in centroid mode on the top 20 precursors pre MS scan using 1 microscan, 17 500 resolution, AGC target of 1E5, maximum injection time of 100 ms and an isolation window of 1.7 *m/z*. Precursors were fragmented by HCD activation with a collision energy of 28%. MS/MS were collected on species with an intensity threshold of 2E4, charge states 1–6 and peptide match off, with masses of interest (*m/z*=783.2653 and 476.1815) on an inclusion list. Dynamic exclusion was set to 20 s.

Sample preparation for LC-MS/MS analysis of >3 kDa fraction of airway fluid

HDI-modified proteins in BAL fluid were identified using proteomic approaches we previously described for human samples (Wisnewski et al., 2004, 2013b). Briefly, the >3 kDa fraction of BAL fluid was concentrated 50-fold using a molecular weight cutoff spin column as described earlier, speedvacced to dryness and then dissolved in 10 μL 8 M urea, 0.4 M ammonium bicarbonate. The proteins were reduced by the addition of 1.0 μL 45mM dithiothreitol (Thermo Fisher Scientific; Waltham, MA), incubated at 37°C for 20 min, alkylated with the addition of 1.0 μL 100mM iodoacetamide (Sigma-Aldrich, St. Louis, MO) and further incubated in the dark at room temperature for 20 min. The urea concentration was adjusted to 2M by the addition of 26–28 μL of water. Samples were then enzymatically digested with 0.2 μg of trypsin (Promega; Madison, WI) at 37°C for 16 h. Digested samples were desalted using C18 Ultra microspin columns (The Nest Group Inc; Southborough, MA) according to the manufacturer's directions, with peptides eluted with 0.1% TFA, 80% acetonitrile. Eluted sample was speedvacced dry, dissolved in MS-loading buffer (2% acetonitrile, 0.2% trifluoroacetic acid), and evaluated using a Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Waltham, MA) to determine protein concentration based on A260/A280. Each sample was then further diluted with MS loading buffer to 0.04 μg/μL and 0.2 μg (5μL) was injected for LC-MS/MS analysis.

LC-MS/MS of >3 kDa fraction of airway fluid

LC-MS/MS analysis was performed on a Thermo Scientific Orbitrap Fusion equipped with a Waters nanoAcquity UPLC system utilizing a binary solvent system (Buffer A: 100% water, 0.1% formic acid; Buffer B: 100% acetonitrile, 0.1% formic acid). Trapping was performed

at 5 μL/min, 97% Buffer A for 3 min using a Waters Symmetry® C18 180 μm × 20mm trap column. Peptides were separated using an ACQUITY UPLC PST (BEH) C18 nanoACQUITY Column 1.7 μm, 75 μm × 250mm (37 °C) and eluted at 300 nL/min with the following gradient: 3% buffer B at initial conditions; 6% B at 5 min; 35% B at 155 min; 85% B at 165 min; 85% B at 180 min; return to initial conditions at 181 min. MS was acquired from the Orbitrap in profile mode over the 350–1550 *m/z* range using quadrapole isolation, 1 microscan, 120 000 resolution, AGC target of 2E5, and a maximum injection time of 60 ms. MS/MS were collected in top speed mode with a 3-s cycle time on species with an intensity threshold of 5E4, charge states 2–8, peptide monoisotopic precursor selection preferred. Dynamic exclusion was set to 20 s. Data-dependent MS/MS were acquired in the Orbitrap in centroid mode using quadropole isolation, HCD activation with a collision energy of 28%, 1 microscan, 60 000 resolution, AGC target of 5E4, maximum injection time of 110 ms.

Identification of HDI-modified peptides

Proteomics data were analyzed using Proteome Discoverer (version 1.3) software (Thermo Fisher Scientific) and searched in-house using the Mascot algorithm (version 2.5.0) (Matrix Science; Boston, MA). The data were searched against a Uniprot database with taxonomy restricted to rabbit. Search parameters used were trypsin digestion with up to two missed cleavages; peptide mass tolerance of 10 ppm; MS/MS fragment tolerance of +0.25 Da; and variable modifications of methionine oxidation, carbamidomethylated cysteine and custom HDI modifications as previously described (Wisnewski et al., 2004, 2013b). Normal and decoy database searches were run with the confidence level set to 95% ($p<0.05$).

Results

Novel approach to study exposure to volatile chemicals

We adapted previously published methods for maintaining spontaneous breathing via endotracheostomy in anesthetized rabbits (Xia et al., 2011) to study lower airway exposure to the occupational allergen HDI. As depicted in Figure 1, rabbits were allowed to free-breathe from a chamber filled with O_2, or O_2 containing HDI vapor for 60 minutes. Given the observed respiration rate (12–20 breaths per/minute) during the exposure period and the average rabbit tidal volume (10 mL/kg) (Garcia-Delgado et al., 2012), the total HDI vapor exposure dose was estimated to be ~30–50 μg. Immediately following exposure, the airways were lavaged through the endotracheal tube.

Identification of HDI reaction products with low (<3 kDa)-molecular-weight components of the airway fluid

The low-molecular-weight fraction of BAL fluid, from $N=2$ each HDI vapor and control exposed rabbits (E1, E2, C1 and C2, respectively), was concentrated by solid-phase extraction and eluted with stepwise increasing concentrations of acetonitrile. Samples were initially analyzed on an Agilent Q-TOF system as described in the methods. Total ion and base peak chromatograms (TICs and BPCs) from LC-MS analyses of paired BAL fluid samples from exposed versus unexposed rabbits were overall qualitatively similar (Supplemental materials, Figure S1). Extracted ion chromatograms (EICs) failed to provide

evidence for unreacted HDI or its hydrolysis product, hexamethylene diamine, for example, $[M+H]^+$ ions with *m/z*'s of 169.09 or 117.14, respectively, in any of the <3 kDa BAL fluid fractions (data not shown). However, $[M+H]^+$ ions with *m/z*'s (783.26 and 476.18) and retention times that matched *in vitro* HDI reaction products with GSH (Wisnewski et al., 2013b), namely bis(GSH)-HDI and mono(GSH)-HDI respectively, were readily observed in BAL fluid fractions from each ($N = 2$) HDI exposed, but not control rabbits (Figure 2, and Supplemental materials Figures S2 and S3). Further, LC-MS/MS analyses of these selected $[M+H]^+$ ions using a Q-Exactive-plus Orbitrap produced HCD fragments consistent with those previously published for bis(GSH)-HDI and mono(GSH)-HDI (Wisnewski et al., 2013b), as shown in Figure 3 and further delineated in supplemental materials Figures S4 and S5. For bis(GSH)-HDI, both the singly and doubly charged species were observed (Figure 3A, and Supplemental materials, Figure S2).

Identification of HDI reaction products with high molecular weight components of the airway fluid

We also analyzed the >3 kDa fraction of BAL fluid from HDI vapor-exposed rabbits using proteomic techniques to identify HDI-modified proteins and their sites of HDI conjugation. LC-MS/MS data identified one peptide with strong evidence of HDI modification in both exposed rabbits, E1 and E2 (Table 1, Figure 4 and Supplemental materials Figures S6–S8). This peptide comprises amino acids 63–70 of uteroglobin (aka Clara or club cell protein), with modification of the lysine at amino acid position 65 (K^{65}) of the mature secreted protein, by partially hydrolyzed HDI (+142.11 kDa). The HDI-modified site is situated near uteroglobin's C-terminus, in close proximity to a disulfide bond that bridges uteroglobin monomers. The side chain of uteroglobin K^{65} has been modeled to protrude from the surface (Bally & Delettre 1989), which may influence susceptibility to HDI conjugation, as shown in Figure 5. Two other sites of uteroglobin modification by HDI are suggested by the data, one in each of the two exposed rabbits, and include modification by partially hydrolyzed HDI (+142.11 kDa) and HDI cross-linking (+168.09 kDa) as shown in Table 1 and Supplemental materials Figures S6 and S7.

In addition to uteroglobin, the LC-MS/MS data provide strong evidence for HDI modification of albumin, on the lysine at amino acid position 525 (K^{525}) of the mature secreted protein, in one of the exposed rabbits. Both the doubly and triply charged species of the tryptic peptide (spanning amino acids 525–534), modified by addition of partially hydrolyzed HDI, were observed (Table 1 and Supplemental materials, Figure S9). The same site has been identified as a dominate reaction site for human albumin with TDI vapor and methylene-diphenyl diisocyanate in vitro (Hettick & Siegel 2012; Hettick et al., 2012).

Discussion

This study pioneers a novel approach to investigate exposure to chemical vapors that cause asthma in the workplace, with a focus on the reaction of inhaled chemical with "self" molecules in the lower airways. To accomplish this goal, we adapted previously described methodology in which rabbits were anesthetized, tracheostomized and allowed to spontaneously free breathe (Xia et al., 2011) through an endotracheal tube. The use of an

endotracheal tube for delivering chemical vapors bypassed the scrubbing effect of the animals' upper airways and provided a ready port for the collection of airway fluid (via lavage) immediately following exposure. Analysis of BAL fluid from HDI-exposed rabbits and comparison with unexposed control animals, using LC-MS and LC-MS/MS techniques, allowed identification of HDI reaction products with several different "self" molecules. The data confirm prior in vitro studies that demonstrate HDI vapor reactivity with GSH and albumin (Wisnewski et al., 2004, 2013b) and identify uteroglobin as another possible target for HDI modification *in vivo*.

Identification of GSH-HDI reaction products *in vivo* supports prior *in vitro* studies demonstrating HDI binding the reactive thiol of GSH under mixed phase (vapor/liquid) conditions, as exists at the air/fluid interface of the airways (Wisnewski et al., 2013b). GSH is a major anti-oxidant of the lower airways and *S* – GSH conjugation is an important step in the metabolism and excretion of many compounds (Cantin et al., 1987; Sipes et al., 1986). The process is usually enzyme (glutathione *S*-transferase) dependent; however, isocyanates may directly conjugate with GSH via a nucleophilic addition mechanism (Day et al., 1997; Ketterer, 1982; Reisser et al., 2002). GSH-HDI conjugates are cleaved by gamma glutamyl transpeptidase in vitro (Wisnewski et al., 2016), the first step in the mercapturic acid pathway, and GSH-monoisocyanates are excreted as their corresponding mercapturic acids *in vivo* (Slatter et al., 1991), suggesting a potentially protective role for GSH against HDI exposure.

The finding of HDI vapor conjugation to albumin *in vivo* supports the overwhelming number of studies demonstrating albumin's importance as a carrier protein for HDI immune recognition, and the use of diisocyanate-albumin adducts as exposure biomarkers (Sabbioni et al., 2012; Wass & Belin, 1989; Wisnewski et al., 2004). Identification of K^{525} of albumin as a binding site for HDI *in vivo* is consistent with multiple *in vitro* studies identifying this site as a preferred diisocyanate target under dose-limiting conditions (Hettick & Siegel, 2012; Hettick et al., 2012; Wisnewski et al., 2013b). The HDI-modified rabbit albumin tryptic peptide observed *in vivo* here is identical to the corresponding HDI-human albumin tryptic peptide observed *in vitro* (Wisnewski et al., 2013b), suggesting its potential as a biomarker for occupational exposure surveillance. The reason HDI-albumin reaction products were identified in only one of the two exposed rabbits is unclear, but may be related to the limited exposure time (one hour)/dose.

Uteroglobin as a reaction target for inhaled HDI vapor is a novel finding of the present study. Rabbit uteroglobin is the homolog of human secretoglobin family 1A member 1 (SCGB1A1) and is commonly referred to by a variety of different names (Clara or club cell protein, CC10, CC16, blastokinin or polychlorinated biphenyl-binding protein) depending upon species/tissue source (Mantile et al., 1993; Mukherjee et al., 2007; Wolf et al., 1992). SCGB1A1 is relatively abundant in the airways, where it is secreted by nonciliated cells that specialize in chemical metabolism (Mukherjee et al., 2007). Polymorphism in human SCGB1A1 is associated with reduced levels of protein and increased likelihood of developing asthma (Candelaria et al., 2005; Ku et al., 2011; Laing et al., 2000; Taniguchi et al., 2013). Of particular relevance to the present study are findings (Kultz et al., 2015) demonstrating uteroglobin as a reaction target for naphthalene, a xenobiotic metabolically

activated within Clara cells. Kultz et al suggest chemical modification of proteins such as uteroglobin, which normally exhibit anti-inflammatory activity (Miele et al., 1987; Mukherjee et al., 2007; Ray et al., 2006; Shijubo et al., 2003; Vasanthakumar et al., 1988), may be key to the pathophysiology associated with certain xenobiotic exposures (Kultz et al., 2015). Further investigation, beyond the scope of this study, will be needed to determine if human uteroglobin is also susceptible to HDI modification *in vivo*, and if so, its potential relevance to occupational exposure and/or disease pathogenesis.

This study's ready identification of GSH-HDI reaction, but limited HDI-protein reaction products warrants further discussion given the classical view of HDI (and other diisocyanates) as haptens that target proteins. The findings may be technical, related to the low total exposure dose, analytical methodology/detection limits, or the focus on BAL fluid versus proteins that are tissue-bound, phagocytized, exchanged with blood/lymph or otherwise removed from the air space. Alternatively, the data might indicate a more primary, and potentially complex role for GSH in response to exposure (Wisnewski et al., 2013a). Under normal conditions, airway fluid GSH levels are relatively high (~100 μM), while protein levels are low compared to tissue or blood levels (Bartlett et al., 2013; Cantin et al., 1987; Grigg et al., 1996). Thus, GSH in airway fluid may compete with proteins for HDI reactivity, thereby preventing antigenic (or other pathogenic) consequences of protein modification. However, the thiocarbamate linkages of GSH with HDI (and other isocyanates) are reversible under the appropriate conditions (pH, temperature) and can mediate stable cabamylation (transfer of HDI) of specific functional groups of host proteins (Brown et al., 1987; Day et al., 1997; Wisnewski et al., 2013b). Thus, GSH reactivity with HDI may initially protect proteins and other vulnerable self-molecules of the airspace from chemical adduction (Brown et al., 1987; Wisnewski et al., 2005) but ultimately allow deeper tissue penetration of the chemical in a reversibly reactive form, possibly potentiating its pathogenicity (Wisnewski et al., 2013a, 2015).

The strengths and weaknesses of the present study should be recognized in interpreting the findings. The strengths include precise LC-MS/MS techniques and discovery science approaches to validate hypotheses based on prior in vitro findings and to identify previously unrecognized targets of HDI reactivity *in vivo*. Weaknesses include the study's exploratory nature, assessing only $N = 4$ animals total and the experimental design, limited to short term exposures and bypassing possibly relevant interactions in the upper airways. The HDI exposure concentrations were relatively high to facilitate chemical detection (despite limited exposure duration) but were not unlike acute peak exposures, such as might occur during an accident or spill, which have been suggested as a crucial factor in the induction of diisocyanate-specific chemical respiratory allergy (Bernstein et al., 1993; Leroyer et al., 1998). An additional weakness of the present study is the certainty of the structures proposed for the HDI reaction products (with GSH, albumin and uteroglobin) based exclusively on tandem LC-MS/MS. The sample size of the rabbit BAL fluid prevented purification and orthogonal verification by ^{1}H- and ^{13}C-NMR, as suggested by the American Chemical Society and Royal Society of Chemistry for defining new compounds (ACS, 2017; RSC, 2017). Finally, it should be noted the present study focused on monomeric HDI vapor and extension of the present findings to other industrially used diisocyanates remains to be tested.

In summary, we developed an *in vivo* approach to evaluate the reaction products of the occupational allergen, HDI, with "self" molecules in the lower airway tract. The study involved vapor exposure of rabbits through a tracheostomy tube to overcome major limitations of previous animal studies, the scrubbing action of rodent upper airways and unintended delivery of chemical to the gastrointestinal tract. The findings confirm *in vitro* studies demonstrating the reactivity of HDI with GSH across a vapor-/liquid-phase boundary and suggest that GSH represents at least one portal of chemical entry into the lower respiratory tract. The data demonstrate *in vivo* HDI modification of albumin on K^{525}, a site previously identified as a preferred target for diisocyanate conjugation through in vitro exposure dose titration studies (Hettick & Siegel 2012; Hettick et al., 2012). The data also identify a previously unrecognized target for HDI conjugation in the airways, namely uteroglobin, the rabbit homolog of human CC10/CC16, aka Clara or club cell protein (Mantile et al., 1993; Mukherjee et al., 2007). Thus, *in vivo* studies of rabbits exposed to HDI vapor via tracheostomy provide evidence that GSH, albumin and uteroglobin are "self" molecules susceptible to HDI modification upon inhalational exposure. The exposure model may be useful for investigating *in vivo* reaction targets of other chemical vapors inhaled from specific occupational or environmental settings.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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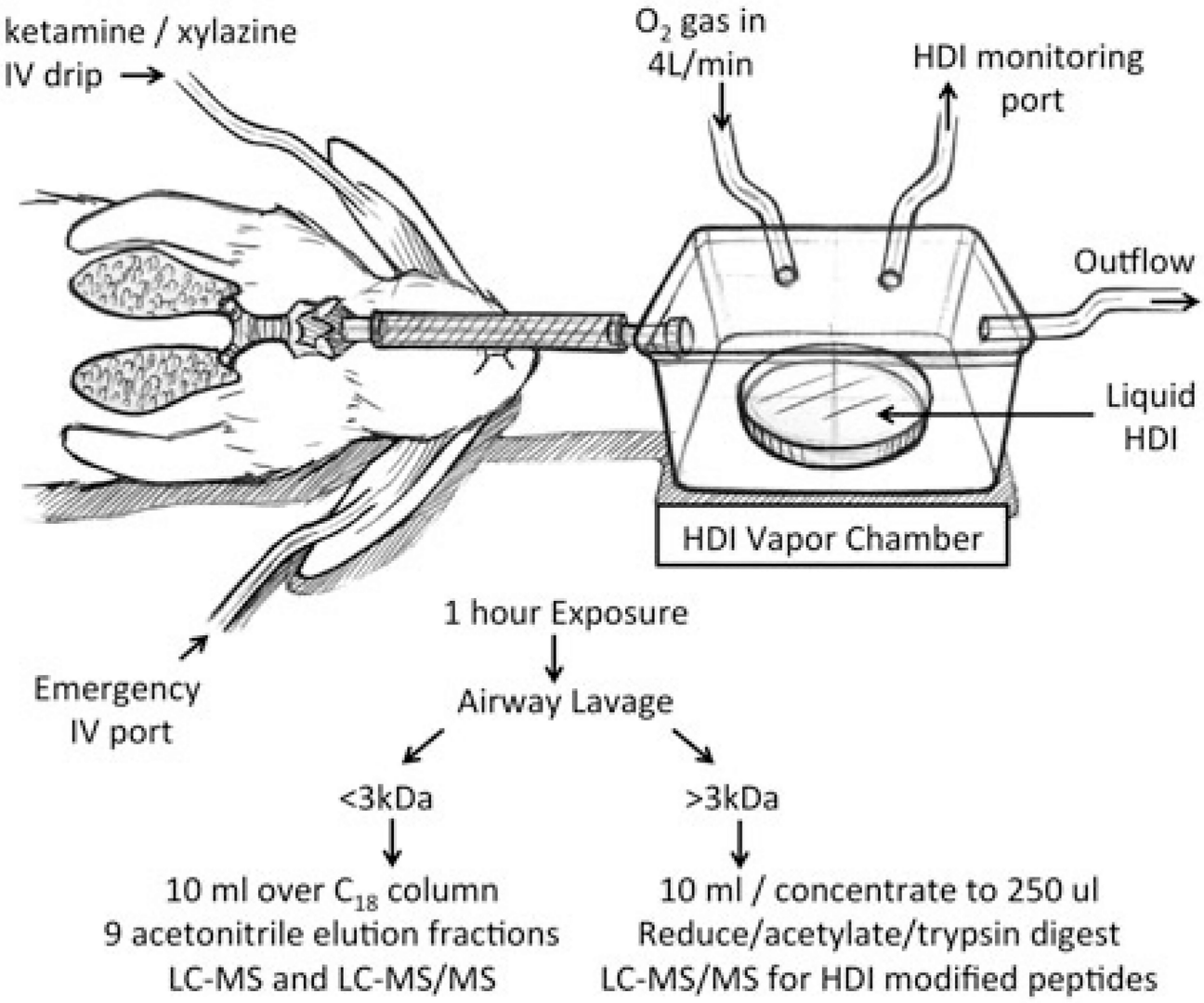

Figure 1.

Schematic representation of experimental setup for *in vivo* HDI vapor exposure of rabbits. Anesthetized rabbits were allowed to spontaneously breathe through an endotracheal tube connected to a chamber filled with either O_2 (control) or O_2+HDI vapor generated by passive diffusion from an open petri dish containing liquid chemical. Endotracheostomy bypasses the scrubbing effect of the upper airways and provides a port for immediate collection of airway fluid (e.g. via lavage) following exposure.

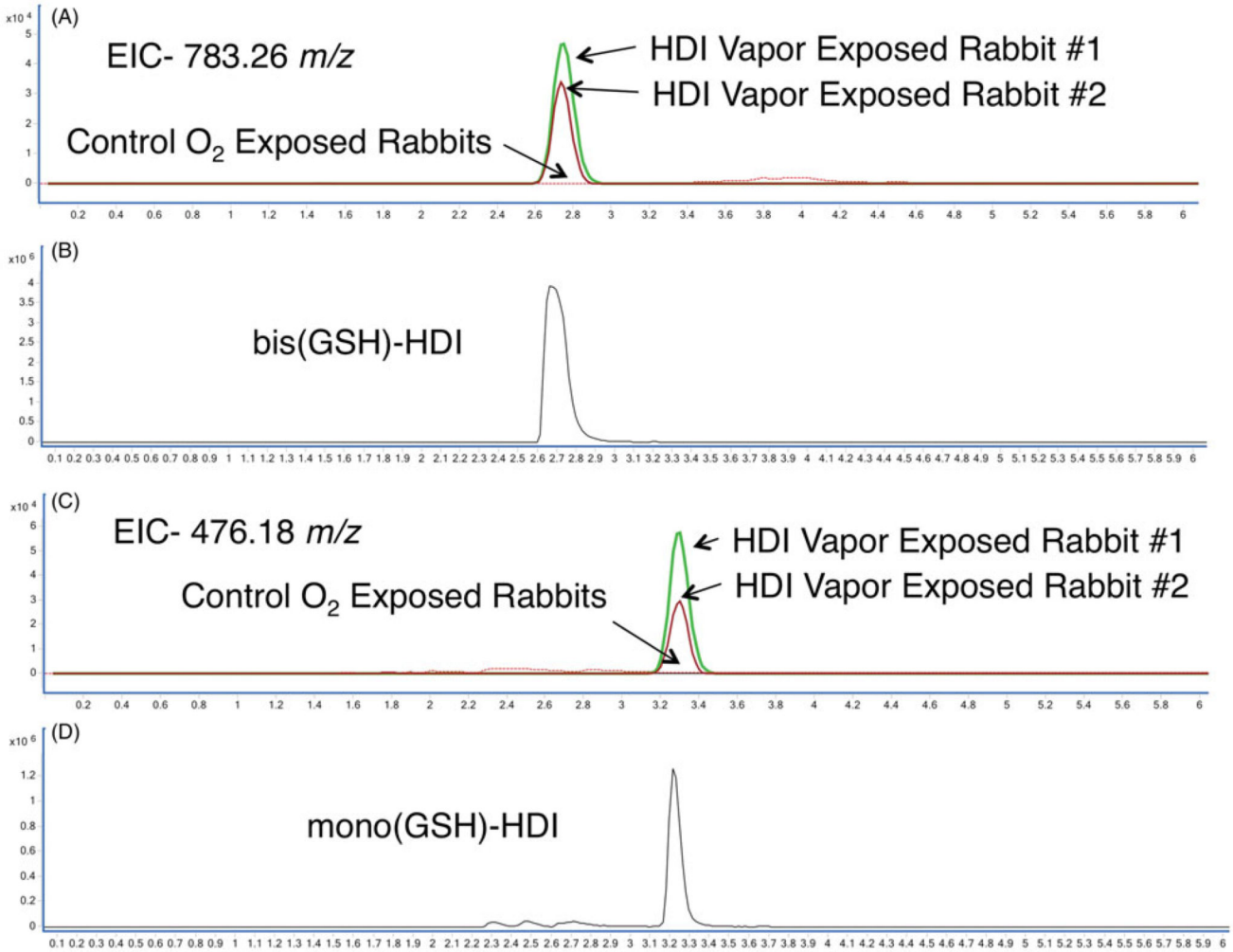

Figure 2.

Extracted ion chromatograms for bis and mono(GSH)-HDI from rabbit airway lavage fluid. Concentrated fractions of BAL fluid from control (dashed lines) and HDI vapor-exposed rabbits (solid lines) were subjected to LC-MS using the Agilent system described in the methods, and EICs were generated for $[M+H]^+$ ions with *m/z*'s previously defined for bis(GSH)-HDI (783.26) and mono(GSH)-HDI (476.18), as shown in Panels A and C, respectively. The defined ions exhibit retention times identical to bis(GSH)-HDI and mono(GSH)-HDI standards generated *in vitro*, Panels B and D, respectively.

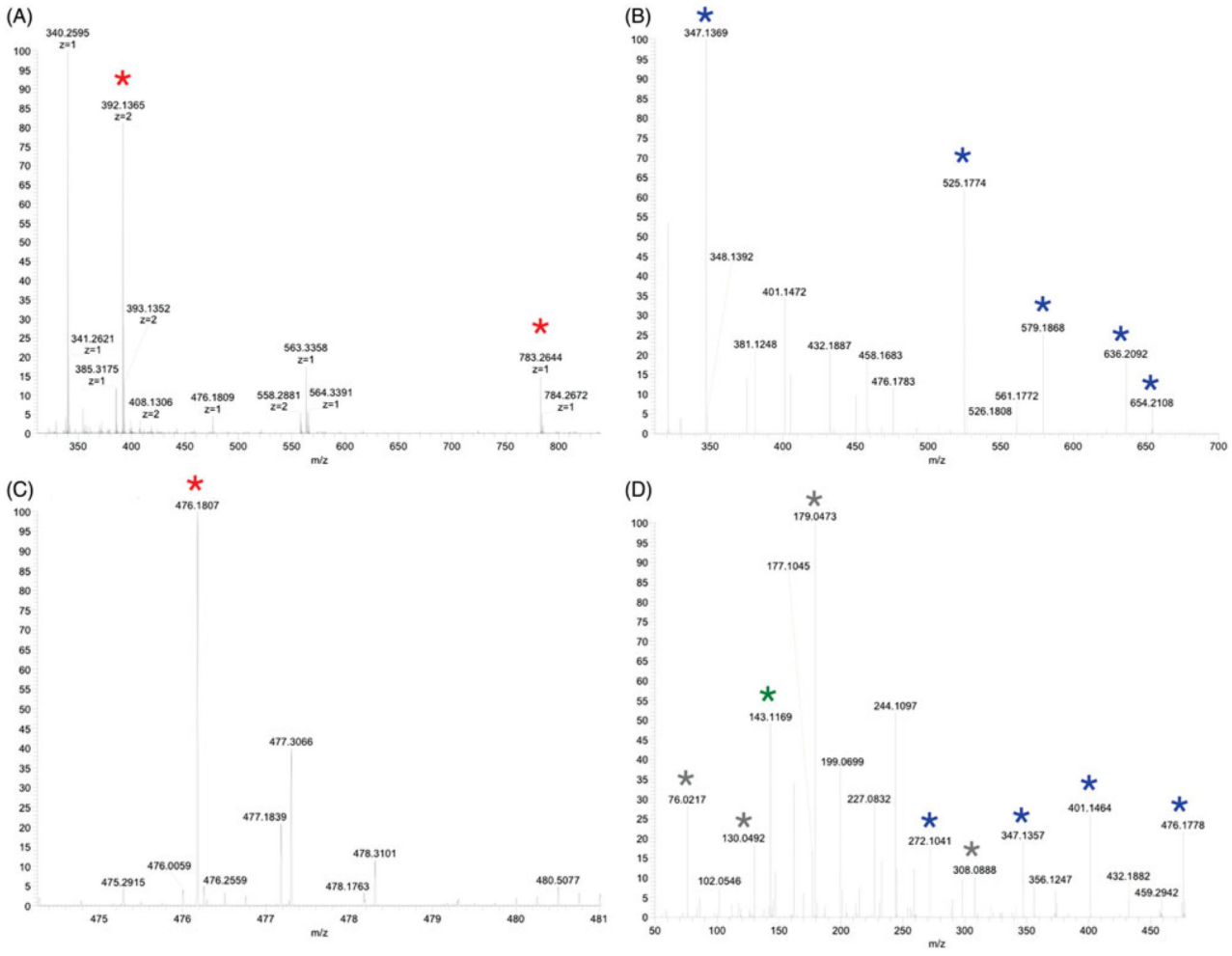

Figure 3.

LC-MS and LC-MS/MS analyses of bis(GSH)-HDI and mono(GSH)-HDI from airway fluid of exposed rabbits. Panel A. Q-Exactive-plus Orbitrap analysis of concentrated BAL fluid from HDI-exposed rabbit, eluting off the LC column at 31.11 min. Singly and doubly charged ions with *m/z*'s matching bis(GSH)-HDI are highlighted with a red asterisk*. Panel B. Q-Exactive-plus Orbitrap MS/MS HCD fragmentation pattern of the 783.26 *m/z* $[M+H]^+$ ion; consistent with that previously reported for bis(GSH)-HDI. Blue asterisks* correspond to expected HCD fragments of bis(GSH)-HDI as delineated in supplemental materials, Figure S4. Panel C. Q-Exactive-plus Orbitrap analysis of concentrated BAL fluid from HDI exposed rabbit eluting off LC column at 37.11 min. The $[M+H]^+$ ion with the *m/z* (476.18) expected for mono(GSH)-HDI is highlighted with a red asterisk*. Panel D. Q-Exactive-plus Orbitrap MS/MS HCD fragmentation pattern of the 476.18 *m/z* $[M+H]^+$ ion, consistent with that previously reported for mono(GSH)-HDI. Blue asterisks* correspond to expected HCD fragments of mono(GSH)-HDI, gray asterisks* correspond to GSH fragments, and the green asterisk* is consistent with the expected *m/z* for the $[M+H]^+$ ion of partially hydrolyzed HDI, as delineated in supplemental materials, Figure S5.

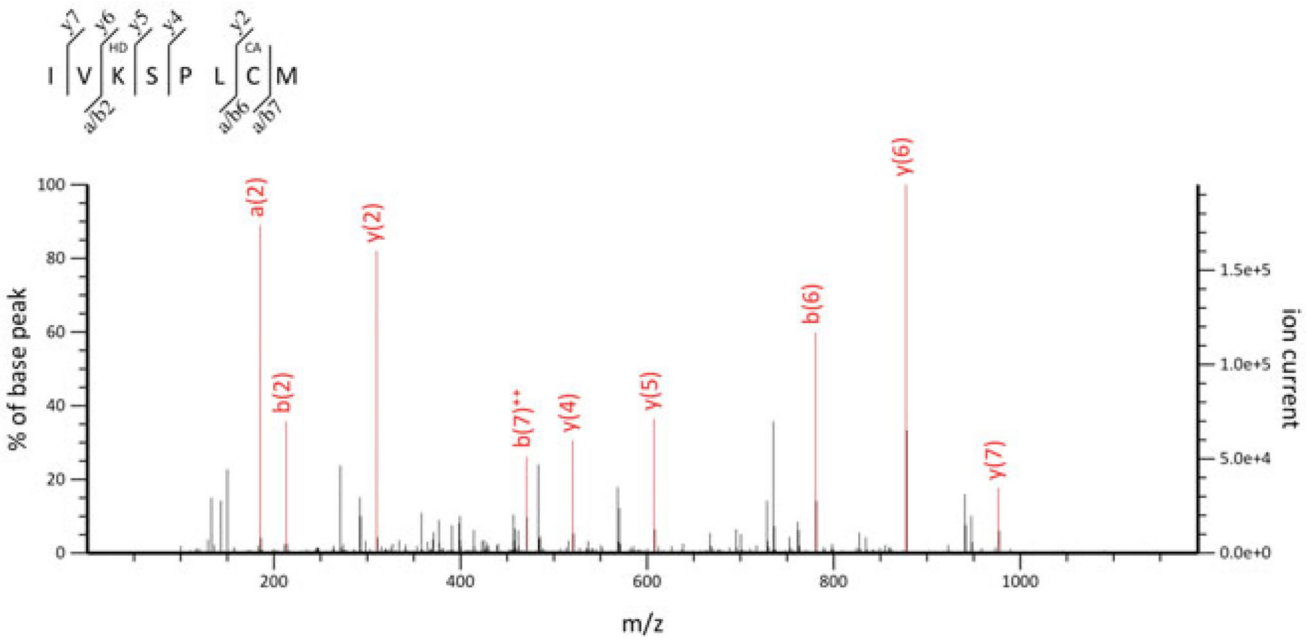

Figure 4.

MS/MS analysis of HDI modified uteroglobin peptide. MS/MS HCD pattern for ion with *m/z* matching that of uteroglobin peptide modified by addition of partially hydrolyzed HDI (+142.1106 amu) on Lys^{65} of the mature protein (plus carbidomethylation of cysteine during sample preparation). Nine of the expected HCD fragment ions are highlighted in red (for additional information see supplemental materials, Figure S8).

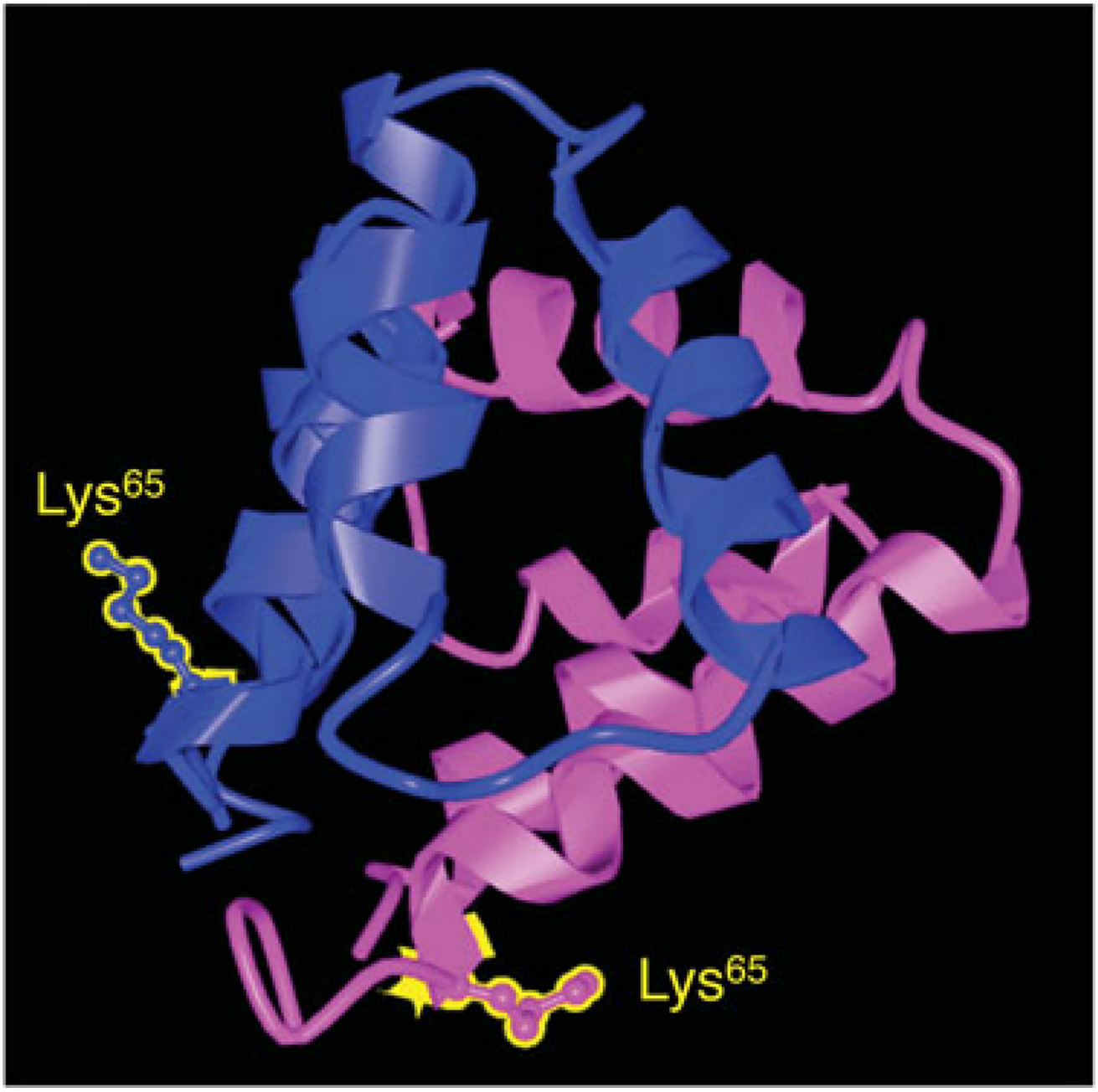

Figure 5.

Molecular model highlighting site of HDI addition to rabbit uteroglobin. HDI modified Lys^{65} residues are highlighted yellow in the predicted molecular model of uteroglobin in its native state, dimerized by disulfide linkage in the C-terminus (Bally & Delettre, 1989).

Table 1

Summary of proteomic data on rabbit airway peptides modified by HDI based on LC-MS/MS analysis.

Protein name	Rabbit	Conjugation Site*	Peptide seq	Amino acids*	Score	*p* value	HDI mod	Other mod**	*m/z*	Charge	Ion Mass (Obs)	Ion Mass (Calc)
Uteroglobin	E1	43	**K**VLDSLPQTTR	43–53	43.55	0.0026	142.1106		467.2804	3	1398.8195	1398.8195
	E1	65	IV**K**SPLCM	63–70	39.94	0.0065	142.1106	C	545.3115	2	1088.6085	1088.6086
	E2	65	IV**K**SPLCM	63–70	45.29	0.0015	142.1106	C	545.3116	2	1088.6087	1088.6086
	E2	65	IV**K**SPLCM	63–70	35.01	0.019	142.1106	C/M	553.3101	2	1104.6057	1104.6035
	E2	65	IV**K**SPLCM	63–70	32.46	0.04	168.0899	C/M	566.2985	2	1130.5825	1130.5828
	E2	62	LTE**K**IVK	59–65	33.02	0.013	168.0899		499.8157	2	997.6168	997.6172
	E2	62	LTE**K**IVK	59–65	26.28	0.013	142.1106		486.8624	2	971.6383	971.6379
Albumin	E1	525	**K**QTALVELVK	525–534	51.42	0.00018	142.1106		635.9086	2	1269.8026	1269.802
	E1	525	**K**QTALVELVK	525–534	45.32	0.00075	142.1106		424.2747	3	1269.8022	1269.802

*Represents amino acid numbering of mature protein, after cleavage of signal/leader sequence for uteroglobin (21 aa) or albumin (24 aa).

**Other modifications C=carbamidomethylation of cysteine, M=oxidation of methionine.

HDI mod indicates increase of 168.0899 or 142.1106 mass units due to modification by HDI or partially hydrolyzed HDI.

Bold underline indicates site of HDI modification.

(Obs)=observed ion mass, all of which are within 0.5 ppm of the (Calc)=calculated or expected ion mass.